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14. ABSTRACT

In year 3, we took a turn and explored into the mechanism behind metformin inhibitory effect on prostsate cancer cells. In our hands, it was clearly shown that metformin treatment causes AR degradation via ubiquitin degradation. The E3 ligase Skp2 is one of the contributing enzyme for this proteasome degradation. We continued our studies with the in vivo orthotopic GRP-Pro CRPC model. Anti-androgen enzalutamide alone is not the most effective therapy for this neuropeptide-mediated aberrant AR activation model. No tumor growth or PSA production was inhibited. On the other hand, metformin alone is able to sequester almost 50% of tumor growth. Combination did not add on much more effect. Lastly, with the CWR22 xenograft model, a well-planned and exhausting effort of drug dosing was abated due to the failure of tumor recurrence after castration. We will revisit this experiment in the no-cost extension period to complete the entire project.

15. SUBJECT TERMS
Castration resistant prostate cancer, autophagy, enzalutamide, metformin, ubiquitination degradation

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Introduction:

We have completed the majority of this study and the results proved that autophagy is the escape mechanism for tumor cells when challenged by small molecule inhibitors such as Src inhibitor saracatinib or the androgen receptor signaling inhibitor enzalutamide. When stresses such as metabolic and genotoxic stress caused by drug treatments, AMPK is activated and mTOR signaling pathway is suppressed to lead to autophagy [1-5]. Knocking down AMPK with siRNA reversed the survival mechanism and led cells to undergo apoptosis. Survival mechanisms elicited by CRPC C4-2B cells when treated with Enza may be blocked by inhibiting autophagy with clomipramine (CMI) [6, 7] and metformin (Metf) [8-10]. Combination of Enza with saracatinib and autophagy modulators significantly reduced cell proliferation in the LNCaP GRP CRPC model. We also reported the effect of saracatinib in preventing CRPC progression using CWR22 xenograft model. In this past year, we extended the study on the effect of comibination of enzalutamide and metformin on LNCaP GRP tumor growth. On the spin-off, we delineated the possible mechanism of metformin in inhibiting prostate cancer progression via ubiquitin degradation of AR.

Keywords: Castration resistant prostate cancer, autophagy, enzalutamide, metformin, ubiquitination degradation

Overall Project Summary:

Metformin causes AR degradation via Skp-2 mediated ubiquitination

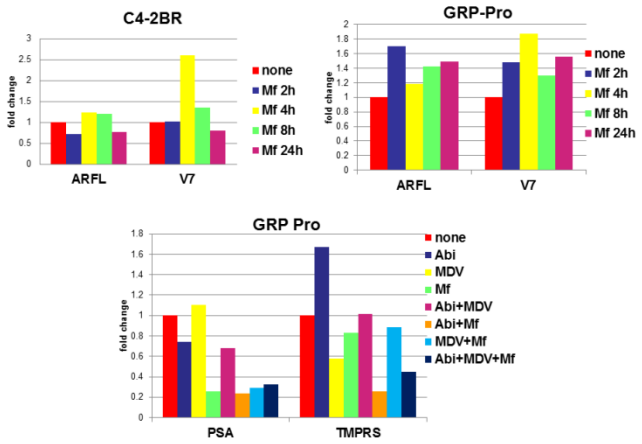

Figure 1. Cells were treated with 0.5 mM of metformin for indicated time or with various drug combinations for 48 hours. Total RNA were extracted. RT-qPCR quantitation of full-legth and variant AR and two AR responsive genes, PSA and TMPRSS2 were performed.

were extracted and real-time RT-PCR was performed to examine the expression levels of full-length AR and the AR-V7 variant. No significant change of the full-length AR level was detected. GRP Pro cells treated with abiraterone (10 µM), enzalutamide (10 µM), metformin (0.5 mM) or the combinations for 48 hours. Total RNAs were extracted and real-time RT-PCR was performed for the expression of PSA and TMPRSS2, both AR surrogate markers. The levels

Per the report by Shen et al. [11], activation of AMP-activation protein kinase (AMPK) by metformin causes decrease of AR protein levels via suppression of AR mRNA expression and promotion of AR degradation. We examined the AR mRNA expression under metformin treatments over a time course and combinations with other drugs. C4-2B MDVR and GRP Pro cells were treated with 0.5 mM of Metformin for 0, 2, 4, 8, 24 hours. Total RNAs

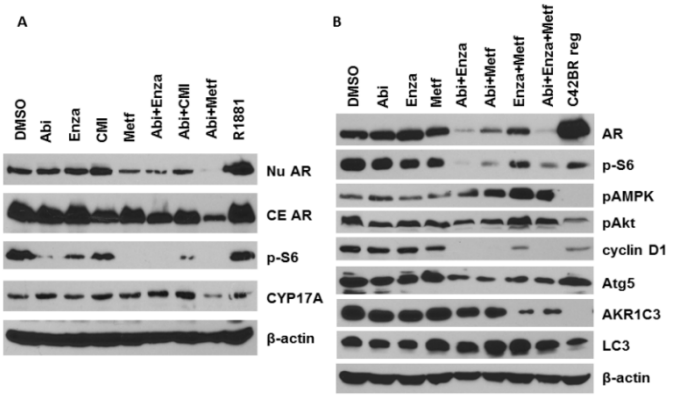

Figure 2. A. LNCaP GRP-Pro cells were treated with DMSO, Abi, Enza, CMI, Metf, Abi+Enza, Abi+CMI, Abi+Metf or R1881 for 72 hours and proteins were analyzed by gel electrophoresis. Decreased nuclear and cytocolic AR levels were detected in samples treated with metformin. B. Similar treatments but in CS medium were analyzed by immunoblotting. Degradation of AR was apparent in all of the combination treatment groups. C4-2BR grown in reg FBS medium was used as control.

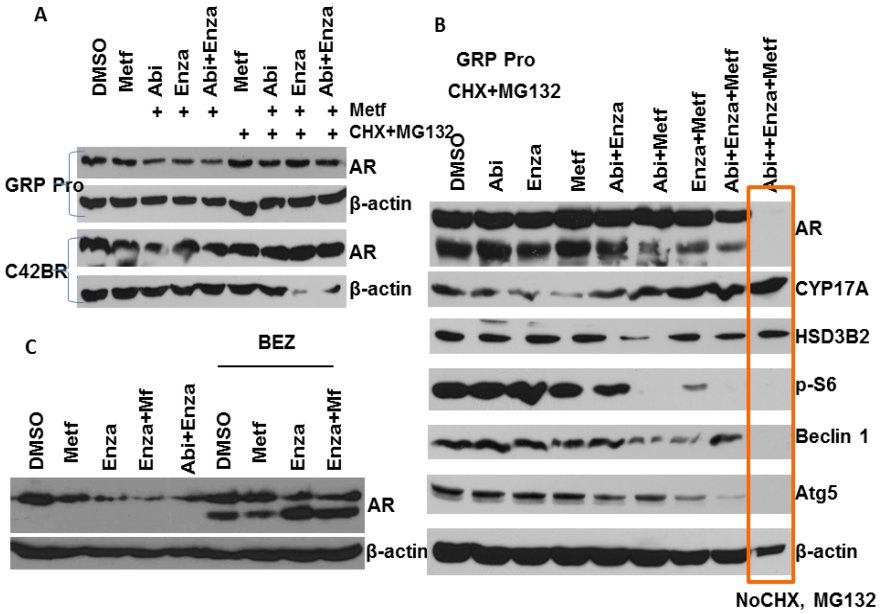

Figure 3. A. LNCaP GRP-Pro and C4-2B+R cells were treated with DMSO, Metformin alone, in combination with abiraterone, enzalutamide or Abi+Enza for 48 hr in the absence or presence of cycloheximide and MG132. Western blotting analysis showed the contents of AR and β-actin. AR degradation was detectable when cells were treated with metformin and CRPC therapeutic drugs, which was inhibited by proteasome inhibitor MG132. B. LNCaP GRP-Pro cells were pre-treated with cyclohexmide, followed by MG132 in couple with DMSO, Abi, Enza, Metf, Abi+Enza, Abi+Metf Enza+Metf, Abi+Enza+Metf for 48 hours. The last lane showed GRP-Pro cells treated with Abi+Enza+metf but no CHX nor MG132. All full-length and truncated AR were preserved in the presence of the proteasome inhibitor compared to the total disappearance of signal in the last lane. C. Addition of Bortezomib (BEZ), a specific proteasome inhibitor also prevented AR degradation caused by drug treatments.

of both genes were reduced when cells treated with metformin or combination drugs (figure 1). We have also observed the change in AR protein levels when cells were treated with metformin alone or in combination with other drugs. Significant loss of full-length AR was observed when cells GRP Pro cells were treated with the combinations of Abi+Enza, Abi+Metf and Abi+Enza+Metf (Figure 2A). The nuclear portion of AR was affected with metf treatment alone, and diminished to very low extent when cells treated with Abi+Metf (Figure 2B). Cells underwent some degree of autophagy when treated with both anti-androgen drugs as levels of phospho-S6 and Atg5 decreased and phospho-AMPK increased. However, the level of LC-3 I remained fairly steady with only marginal production of LC-3 II, so did the p-Akt. Because of the relative stable AR expression and yet drastic reduction of AR protein levels in response to metformin, we suspected post-translational modification, namely protease degradation.

The loss of AR in the presence of metformin and other drugs was prevented by prior supplementation of cycloheximide and MG-132, a universal proteasome inhibitor (Figure 3A). In both C4-2B MDVR and GRP Pro cells, AR levels were preserved when the proteasome activity was inhibited. Full-length AR remained stable in cells pre-treated with cycloheximide and MG-132 in all conditions compared to the one without the proteasome inhibitor (last lane, Figure 3B) in GRP Pro cells. However, the levels of AR variants still suffer some degree of loss, suggesting further action in the cells. Cells still turned to autophagy as a survival mechanism even when the AR levels were

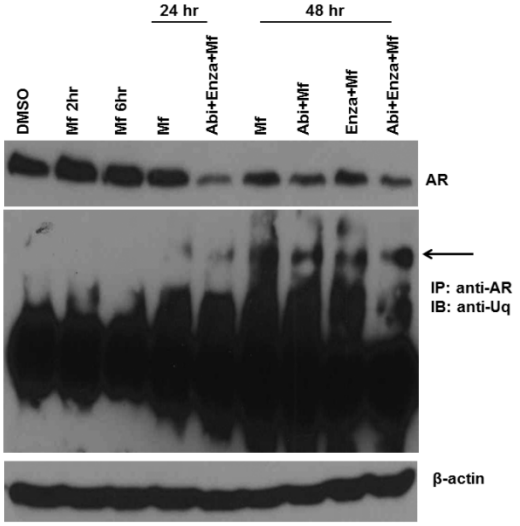

Figure 4. C4-2B cells were treated with various reagents for different periods of time as indicated. Cell lysates were subjected to immuno-precipitation with anti-AR antibody and followed by protein G agarose. The pull-down proteins were analyzed and probed with anti-ubiquitin antibody. AR degradation was apparent in lanes with >24 hours of metformin and combination treatments. Bands at the position of AR MW were detected, suggesting co-IP of AR and ubiquitin.

maintained as the autophagy markers p-S6, beclin1 and Atg1 still responded accordingly. Cells were pre-treated with another proteasome inhibitor, Bortezomib (Bez) in the same treatments. Those samples pre-treated with Bez showed conservation of both full-length and variant AR even in the presence of metformin or enza+metformin (Figure 3C). These results supported that the decrease of AR protein levels was due to protease degradation.

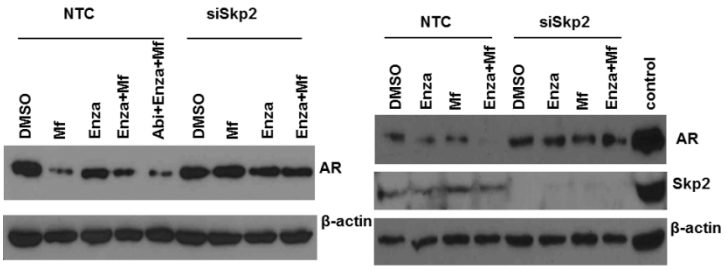

Figure 5. C4-2B and GRP Pro cells were transfected with siRNA against E3 ligase Skp2 or non-target control, followed by treatments with DMSO, Enza, Metf or Enza+Metf, Abi+Enza+Metf for 48 hours. Cell lysates were analyzed for AR protein levels. AR, Skp2 and actin levels were shown to demonstrated AR degradation, Skp2 knockdown and loading control, respectively.

treatments for 48 hours. Cells in the control group transfected with non-target control siRNA showed AR degradation with drug treatments (Figure 5). However, AR levels remained stable in those transfected with siSkp2, suggesting Skp2 could be one of the E3 ligases responsible for AR degradation in these conditions.

With the C4-2B orthotopic tumor results published in our previous study, addition of metformin effectively blocked the autophagy survival mechanism tumor cells used when treated by anti-androgen enzalutamide. We took representative samples from this study and extracted total RNA to examine the levels of some AR downstream molecules and intracrine androgen synthetic enzymes. Both PSA and TMPRSS2 levels decreased drastically with either enzalutamide or metformin alone.

Since AR degradation may be inhibited by proteasome inhibitor MG132, we attempted to show ubiquitination of AR via co-immunoprecipitation of AR and ubiquitin. LNCaP C4-2B cells were treated with metformin alone or in combinations with anti-androgens for different time intervals. Full-length AR decreased with 48 hours of metformin treatment but was aggravated by combinations of enzalutamide, abiraterone or both (Figure 4). The detection of ubiquitin co-IPed with AR was intensified with time and combined drug treatments. Involvement of ubiquitin E3 ligases have been implied in CRPC [12]. Among them, Skp2 has been reported to regulate AR level through ubiquitin degradation [13, 14]. We therefore attempted to knock down Skp2 before metformin treatments to see if AR level was retained. C4-2B and GRP Pro cells were transfected with siSkp2, followed by metformin, enzalutamide or combination

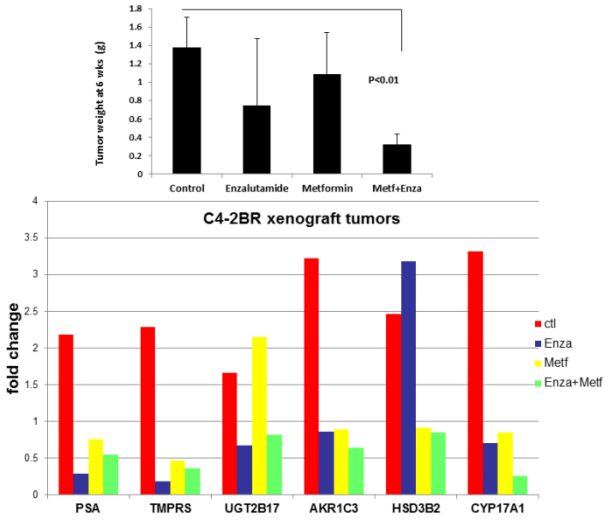

Figure 6. Enzalutamide and metformin monotherapies did not inhibit the orthotopic tumor growth significantly. However, combination of Enza and Metf treatment significantly reduced tumor weights when compared to the control group. Total RNAs extracted from representative tumors from control, Enza, Metf and Enza+Metf groups were subjected to RT-qPCR for the genes indicated. Most of the expression went down with just a single treatment and the combination ensured the low expression for all genes.

Testing the combination of enzalutamide and metformin on LNCaP GRP-Pro orthotopic mouse model

We have reported the effect of specific Src inhibitor saracatinib, enzalutamide and the combination on the tumor progression of orthotopic CRPC LNCaP GRP-Pro model. Because of the specific driving force for this CRPC model, aberrant AR activation through neuropeptide (GRP) mediated signaling through the tyrosine kinases Src-Etk-Fak complex [15], the combination of saracatinib and enzalutamide was very effective in inhibiting tumor growth. Since autophagy mechanism is the focus of this funded project, we continued to test whether combination of autophagy modulator metformin with enzalutamide will also benefit the tumor treatment.

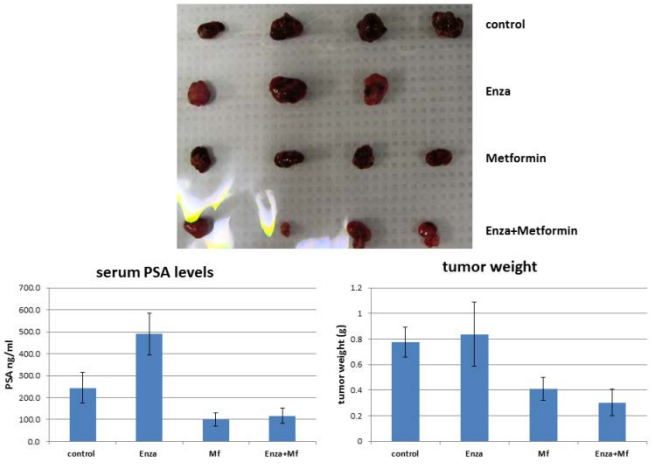

Figure 7. LNCaP GRP-Pro orthotopic tumors were harvested from castrated SCID mice. Serum PSA levels and tumor weight were measured and tallied, and the mean values of each group were graphed with standard errors.

Male SCID mice were castrated and orthotopically implanted with two millions of GRP-Pro cells. Serum PSA levels were measured 12 days later to confirm tumor take. Animals were randomly divided into 4 groups and treated with enzalutamide (25 mg/kg, p.o. daily), metformin (300 mg/kg, p.o. daily), combination or buffer only. After 30 days of treatments, animals were sacrificed. Blood and tumor were collected for analysis. The result (Figure 7) was consistent with previous findings that enzalutamide alone did not inhibit tumor growth. Serum PSA and tumor weight from the Enza group scored the highest among all. Metformin alone was powerful enough to bring down the serum PSA level and tumor weight by almost half. However, addition of enzalutamide with metformin did not completely abolish tumor growth. The benefit was minimum as the decrease in tumor weight from the combination group was only by 25% when compared to metformin alone. Decrease from neither group scored a *p* value for significant difference. This is probably due to the specific neuropeptide-mediated AR activation of the GRP-Pro line. Enzalutamide is never the ideal treatment drug. Although metformin may cause AR degradation, especially over the long-term treatment, AR activation via Src-Etk-Fak signaling is always present from GRP binding to its G protein-coupled receptor. The result of this model serves as an example for the need of precision medicine. If tyrosine kinase activation/up-regulation is the main force for the disease, a specific inhibitor to block that signaling pathway is required. The immunohistochemical staining of representative tumors from each group showed that Ki67 decreased drastically in the Metf treatment, and so did in the Enza+Metf group. The AR staining was still prevalent in the Enza group but reduced profoundly in the Enza+Metf group.

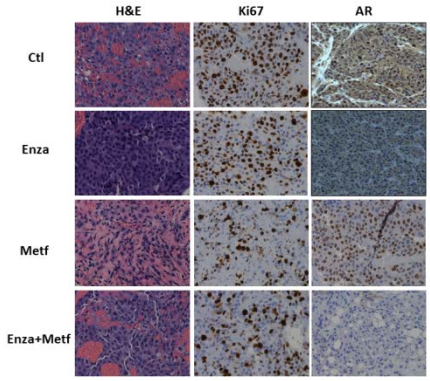

Figure 8. Tumor samples from each treatment groups were section and immunostained with antibodies against AR and Ki67, respectively. Representative views are shown here.

Testing of the combination of Enzalutamide and metformin on the CWR22 xenograft model, incomplete

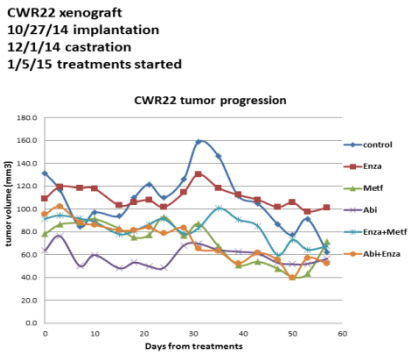

Figure 9. Tumor measurement of CWR22 xenograft mouse study after castration. Tumor sizes eventually dwindled to nothing in almost all mice regardless of treatments.

From the success using the CWR22 xenograft model [16, 17] to mimic tumor recurrence after androgen deprivation therapy in patients last year, we revisited this model using anti-androgens enzalutamide and abiraterone paired with autophagy modulator metformin for treatments. Athymic Nu/Nu male mice were implanted with CWR22 xenograft subcutaneously and allow for tumor progression. After tumor volume reached 100-150 mm^3, mice were surgically castrated. Tumor regression and PSA levels were used to monitor response from castration. Treatments were started 34 days after castration when PSA levels have dropped to minimum. Animals were randomly divided into groups, subjected to no treatment, treatments with enzalutamide (25 mg/kg), abiraterone (200 mg/kg), metformin (300 mg/kg) or combinations, Abi+Enza and Enza+Metf. Tumors were continuously measured biweekly with caliper. Two months after treatment start date, or 3 months after castration, there was still no recurrence of tumor detectable. Treatments were stopped with continual tumor measurement. After 6 months of wait, the control group still showed no sign of tumor regrowth. Out of 42 mice, only 3 showed recurrence of tumor growth. The experiment had to be halted for this low number.

Key Research Accomplishments:

1. Confirmed AR degradation caused by the metformin treatment in both C4-2B and GRP-Pro cells. The extent of degradation aggravated when metformin was combined with enzalutamide or abiraterone. This degradation was possibly mediated by E3 ligase Skp2.
2. In vivo studies with CRPC LNCaP-GRP cells showed some effect of metformin in abrogating tumor growth. However, since enzalutamide could not strategically inhibit the CRPC growth of GPR-Pro cells mediated by neuropeptide-driven AR activation, coupling use of enzalutamide and metformin did not provide any treatment benefit for this particular case.

Conclusion:

In year 3, we took a turn and explored into the mechanism behind metformin inhibitory effect on prostsate cancer cells. In our hands, it was clearly shown that metformin treatment causes AR degradation via ubiquitin degradation. The E3 ligase Skp2 is one of the contributing enzyme for this proteasome degradation. We continued our studies with the in vivo orthotopic GRP-Pro CRPC model. Anti-androgen enzalutamide alone is not the most effective therapy for this neuropeptide-mediated aberrant AR activation model. No tumor growth or PSA production was inhibited. On the other hand, metformin alone is able to sequester almost 50% of tumor growth. Combination did not add on much more effect. Lastly, with the CWR22 xenograft model, a well-planned and exhausting effort of drug dosing was abated due to the failure of tumor recurrence after castration. We will revisit this experiment in the no-cost extension period to complete the entire project.

Publications, Abstacts, and Presentations:

<u>Poster presentation:</u>

Metformin causes AR degradation via Skp2-mediated ubiquitination JC Yang, AC Gao and CP Evans AACR 2015

Inventions, Patents and Licenses: N/A

Reportable Outcomes: None

Other Achievements: None

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Appendices: None